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(54) Title: LIGHT-ACTIVATABLE PLANT PROMOTER

(57) Abstract

A light-activatable plant promoter is provided. This promoter was identified as a promoter sequence for the fructose-1,6-bisphosphatase gene. The promoter can be used to control the expression of genes in photosynthetic tissues.

* See back f page

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LIGHT-ACTIVATABLE PLANT PROMOTER

The present invention relates to a light-activatable plant promoter.

Fructose-1,6-bisphosphatase (FBPase) is a key enzyme in the reductive pentose phosphate pathway of carbon fixation of photosynthesis. The protein is nuclear encoded. The wheat genome contains three copies, one derived from each of the progenitors of hexaploid T. aestivum. FBPase is synthesised as a precursor protein with a transit sequence of about 50 amino acids which is cleaved from the mature protein during transport into the chloroplast.

The enzyme activity of FBPase is light-regulated via the ferredoxin/thioredoxin system. A full length cDNA for wheat FBPase has been isolated (Raines et al, Nucl. Acids Res. 16, 7931-7942, 1988). This provided the first complete amino acid sequence for FBPase. The cDNA clone was also used as a probe to determine steady state levels of FBPase mRNA in wheat leaves during development and also under different light regimes (Raines et al, 1988). These results suggested that FBPase gene activity is responsive to light although other developmental factors appeared also to influence final mRNA levels.

We have now identified a promoter sequence for the FBPase gene. Accordingly, the present invention provides a light-activatable promoter having the sequence:

ATCGATAGGTTCGACAGAAGCTGCCACGTACCTATGAAACTCAATTGATTATTATATCCA
TATTATTGAAGTTTATTTTTGTAGAATGTTATTCAATTCCAGAAGTTGTAAGTTCCGTAA
GACCTGTAATATTGGCCCAATCGAAGACCAACGTTTCTTGAGCTAAATTTAGCTTTTTTT
TTAGAAAGTAATTTAGCTTGCACCCTGGTAAGTGCCACAGAGTGGCACCAATACATGGAA
CTCAAACATTTTTTGTCCCAGGTTTAGTGACGCGATGATGACAGCTTTAGTTGTCAAGCA
TGACAACCTTTTTGAATGGTAAGTTTTACTCTCTTTTTTAGATGACAACTTAGTTTTTAA
AAAGTCAGGCCAAAGTGCTTCGTGACACACGTGTGACACTTATCATTCAGTTTGTCTAAT
TCACATCTAGATATTTTTTAAGGATGTCACATCTAACCTCCCACAAGTATATAATGCATC
AATAAGAAACAAAAAAAACTAGGACAAAAAAATAGACCACAAACAGAGTGAAAATCAGTT
TAGATATGACATAACTATGTCACATTTAGATGTGTCCTAGACAGACCCCTTATCATTTGG
GTTTACCTTTAGTAGCGGGCGTATGCCGCGGCATCAATAAATCATCATCATGTAATGTAT
ATAAGGCGTTTAGAGACTTGACGAAGGTTGTATCTACGATCCACGAAATATCTAATCTCC
AAAGGTAGGTGAATCAGACTGAAGCGAGCGACCACATCCTCACAATTCTGCCCCAATCCA
CACATTCGCCTCCAGCCCTCTCTCAAGCCACACAAACCGAGCCCGGAACCAATGGAACAA
AACAAGAAGCCGGCACCACCACCACGGTG

optionally modified by one or more base substitutions, insertions and/or deletions and/or by an extension at either or each end provided that the thus-modified sequence is capable of acting as a light-activatable promoter.

The invention also provides a DNA fragment comprising such a promoter operably linked to a heterologous gene encoding a protein. Additionally provided is a vector which comprises a heterologous gene encoding a protein under the control of a promoter as above such that the gene is capable of being expressed in a plant cell transformed with the vector. A suitable vector is one in which the promoter is fused directly to the 5'-end of the gene. The vector may

further contain a region which enables the gene and the promoter to be transferred to and stably integrated in a plant cell genome. The vector is generally a plasmid.

Plant cells can be transformed with such a vector. The invention therefore further provides plant cells which harbour a promoter as above operably linked to a heterologous gene encoding a protein. Transgenic plants may be regenerated from such plant cells. A transgenic plant can be obtained which harbours in its cells a promoter as above operably linked to a heterologous gene encoding a protein. Seed may be obtained from the transgenic plants.

The invention further provides a method of producing a desired protein in a plant cell, which method comprises:

(i) transforming a plant cell with a vector according to the invention, the protein encoded by the gene under the control of the said promoter being the desired protein; and

(ii) culturing the transformed plant cell under conditions of light which allow expression of the protein.

The invention additionally provides a method of producing a transgenic plant capable of producing a desired protein, which method comprises:

(i) transforming a plant cell with a vector according to the invention, the protein encoded by the gene under the control of the said promoter being the desired protein; and

(ii) regenerating plants from the transformed cells.

The desired proteins can be isolated from the transformed plant cells obtained by the first method and from the plants cbtained by the second method.

The present promoter is composed of the sequence upstream of the wheat FBPase gene from base -934 to base -54, base 1 being A of the ATG translational start codon for FBPase. The promoter may be obtained by preparing a genomic library of wheat DNA, screening the library for the FBPase gene and digesting the sequence upstream of the wheat FBPase gene with appropriate restriction enzymes. There is a ClaI restriction site at base -934 and a HhaI restriction site at base -54.

Several plasmid vectors have been prepared which contain an upstream sequence of the wheat FBPase gene comprising the promoter sequence from base -934 to base -54. These vectors include p1.8ES. E. coli MC 1022 harbouring p1.8ES was deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, GB on 25 August 1989 under accession number NCIMB 40184. The promoter may be released by ClaI/HhaI digestion of p1.8ES.

The promoter sequence may be modified by one or more base substitutions, insertions and/or deletions and/or by an extension at either or both ends. However, the modified promoter sequence must still be capable of acting as a light-activatable promoter. A shortened promoter sequence from base-497 to base -54 of the upstream sequence of the wheat FBPase gene has been found not be sufficient to direct

expression of a protein at satisfactory levels. Typically there is a degree of homology of at least 60% between a modified sequence and the unmodified natural sequence from base -934 to base -54 upstream of the wheat FBPase gene. The degree of homology may be at least 75%, at least 85% or at least 95%.

A longer promoter sequence may be provided which extends upstream of base -934, for example to another restriction site. An extension upstream of base -934 typically comprises the natural FBPase sequence upstream of base -934. There is an EcoRI site at base -1726 of the upstream sequence of the FBPase gene. A suitable extended promoter therefore has the sequence:

GAATTCTAAGGATGGGCATGGGAGGGGGTGGGGTTTCAACCTGAGGATTTTTTCCAACCT

AAATACAGACTATGCAAACTACCTATTGTGTTTTATTCAAAAATACATCCCTTGCCCAAA

ACGAGGTAGGGCAACACCTATAGATGGCTATGGAAGAAGTTATGGAGTATCATAATATAT

GTTTCATCATATCCTTCCATATGTTATCATGATAACTTTAAAGTCCTGAAATTTGCGATA

TGCACTTGAAGCTCCGCTGTCATTCCCATGGCTTAAAAGTCACTGATTGGAGAGCATGTT

GTGTTTTTGCCCGTAGATCCATTGGCTTTAAGGTTTGATAGGTCATCGCCCTATGCTTTA

ACCTATCATGACAATAGTGGCAACAAATGCGGAAATCCAACTCGGTGCCCAGGCTCATCT

GCTCTCGGTCAGAAAAAAAATCAAAACAAATACTAGAAAAATACAAACCCCGATTTGTCT

TTTTTGCCGAGAGCTACTCAGATGTCCAAATGAGTTGAACTTTAGAACGGACCTACGTAT

CGAATTATCTACCACACATTTTTTTTTAATTTTTCTAGTATTTGTTATGATTTTTTTGTC

AGTGCACCTAAGCCCGGGAGCAGAAACGCCGCGTCCCAACAAATGCTAATGTTACGTTTG

ATTTGGTAAATGGTTCTCTTTGTGTTGTGTATCTTTTGGTTGTATTAGAAATCTTTGTGG

TCCATATGTGCATAATTTGTTCAATAAATCAATAATGTCAGATCGTCCTAAAATAAACTT

GAGAAGAAATTCATCGATAGGTTCGACAGAAGCTGCCACGTACCTATGAAACTCAATTGA

TTATTATATCCATATTATTGAAGTTTATTTTTGTAGAATGTTATTCAATTCCAGAAGTTG

TAAGTTCCGTAAGACCTGTAATATTGGCCCAATCGAAGACCCAAGTTTCTTGAGCTAAAT

TTAGCTTTTTTTTTAGAAAGTAATTTAGCTTGCACCCTGGTAAGTGCCACACGAGTGGCA

CCAATACATGGCAACTCCAAACATTTTTTGTCCCAGGTTTAGTGACGCGATGATGACAGC

TTTAGTTGTCAAGCATGACAACCTTTTTGAATGGTAAGTTTTACTCTCTTTTTTGAGTTT

TTTAGATGACAACTTAGTTTTTAAAAAGTCAGGCCAAAGTGCTTCGTGACACACGTGTGA

CACTTATCATTCAGTTTGTCTAATTCACATCTAGATATTTTTTAAGGATGTCACATCTAA

CCTCCCACAAGTATATAATGCATCAATAAGAAACAAAAAAAACTAGGACAAAAAAATAGA

CCACAAACAGAGTGAAAATCAGTTTAGATATGACATAACTATGTCACATTTAGATGTGTC

CTAGACAGACCCCTTATCATTTGGGTTTACCTTTAGTAGCGGGCGTATGCCGCGGCATCA

ATAAATCATCATGTAATGTATATAAGGCGTTTTAGAGACTTGACGAAGGTTGTATCTACG

ATCCACGAAATATCTAATCTCCAAAGGTAGGTGAATCAGACTGAAGCGAGCGACCACATC

CTCACAATTCTGCCCCCAATCCACACATTCGCCTCCAGCCCTCTCTCAAGCCACACAAAC

CGCAGCCCGGAACCAATGGAACAAAACAAGAAGCCGGCACCACCACCACGGTGC.

This longer sequence may be obtained from a genomic library of wheat DNA as above or by digesting pl.8ES with EcoRI and HhaI. The longer sequence may also be modified by one or more base substitutions, insertions and/or deletions and/or by an extension at either or both ends. Again, such a modified sequence must be capable of acting as a light-activatable promoter. There may be a degree of homology of at least 60%, for example at least 75%, at least 85% or at least 95%, between the modified sequence and the unmodified natural sequence from base -1726 to base -54 upstream of the wheat FBPase gene.

A modified promoter sequence may be obtained by introducing changes into the natural promoter sequence. This may be achieved by any appropriate technique, including restriction of the natural sequence with an endonuclease, insertion of linkers, use of an exonuclease and/or a polymerase and site-directed mutagenesis. A shorter DNA sequence therefore may be obtained by removing nucleotides from the 5'-terminus or the 3'-terminus of the natural promoter sequence, for example using an exonuclease such as BAL 31.

Whether a modified sequence is capable of acting as a light-activatable promoter may be readily ascertained. The modified sequence is placed upstream of a protein coding sequence, such as the bacterial reporter gene β-glucuronidase as in the Example. Tobacco leaf discs can then be transformed. The protein expressed when the

transformed cells are exposed to light is then assayed, in the case of β-glucuronidase as described in the Example.

The promoter may be operably linked to a heterologous gene encoding a protein. The heterologous gene may encode any protein it is desired to express. By "heterologous" is that the gene is not naturally operably linked to the promoter. The gene does not therefore encode wheat FBPase. The protein may comprise a transit peptide sequence at its N-terminus.

The promoter is typically used to control the expression of genes in photosynthetic tissues. The protein whose expression is controlled by the promoter may be a protein encoded by a herbicide-resistance gene or a protein conferring biological control of pests or pathogens. The protein may therefore be an insecticidal protein, such as B. thuringiensis toxin, to give resistance to leaf-eating insects. Other uses to which the promoter may be put are the production of viral coat proteins to protect against viral infection, the production of high value proteins such as pharmaceuticals and the production of proteins to alter taste or nutritive value of forage grasses, etc.

The promoter sequence may be fused directly to a heterologous gene or via a linker. The linker sequence may comprise an intron. Excluding the length of any intron sequence, the linker may be composed of up to 45 bases, for example up to 30 or up to 15 bases. We have found that no protein was expressed, however, when a gene encoding

β-glucuronidase was fused to the promoter via the sequence from base -53 to base 129 of the wheat TPase gene.

DNA fragments and vectors can be prepared in which the promoter is operably linked to a heterologous gene. The fragments and vectors may be single or double stranded. Plant cells can be transformed by way of such fragment directly or by way of such a vector. The vector incorporates the heterologous gene under the control of the promoter. The vector contains regulatory elements capable of enabling the gene to be expressed in a plant cell transformed with the vector. Such regulatory elements include, besides the promoter, translational initiation and/or termination sequences. The vector typically contains too a region which enables the chimaeric gene and associated regulatory control elements to be transferred to and stably integrated in the plant cell genome.

The vector is therefore typically provided with transcriptional regulatory sequences and/or, if not present at the 3'-end of the coding sequence of the gene, a stop codon. A DNA fragment may therefore also incorporate a terminator sequence and other sequences which are capable of enabling the gene to be expressed in plant cells. An enhancer or other element able to increase or decrease levels of expression obtained in particular parts of a plant or under certain conditions may be provided in the DNA fragment and/or vector.

Transformed cells are selected by growth in an

appropriate medium. Plant tissue can therefore be obtained comprising a plant cell which harbours the heterologous gene under the control of the promoter , for example in the plant cell genome. The gene is therefore expressible in the plant cell. Plants can then be regenerated which include the heterologous gene and the promoter in their cells, for example integrated in the plant cell genome, such that the gene can be expressed. The regenerated plants can be reproduced and, for example, seed obtained.

A preferred way of transforming a plant cell is to use Agrobacterium tumefaciens containing a vector comprising the promoter operably linked to the heterologous gene. A hybrid plasmid vector may therefore be employed which comprises:

(a) the heterologous gene under the control of the promoter and other regulatory elements capable of enabling the gene to be expressed when integrated in the genome of a plant cell;

(b) at least one DNA sequence which delineates the DNA to be integrated into the plant genome; and

(c) a DNA sequence which enables this DNA to be transferred to the plant genome.

Typically the DNA to be integrated into the plant cell genome is delineated by the T-DNA border sequences of a Ti-plasmid. If only one border sequence is present, it is preferably the right border sequence. The DNA sequence which enables the DNA to be transferred to the plant cell

genome is generally the virulence (vir) region of a Ti-plasmid.

The heterologous gene and its transcriptional and translational control elements, including the promoter, can therefore be provided between the T-DNA borders of a Ti-plasmid. The plasmid may be a disarmed Ti-plasmid from which the genes for tumorigenicity have been deleted. The gene and its transcriptional and control elements, including the promoter, can, however, be provided between T-DNA borders in a binary vector in trans with a Ti-plasmid with a vir region. Such a binary vector therefore comprises:

(a) the heterologous gene under the control of the promoter and other regulatory elements capable of enabling the gene to be expressed when integrated in the genome of a plant cell; and

(b) at least one DNA sequence which delineates the DNA to be integrated into the plant genome.

Agrobacterium tumefaciens, therefore, containing a hybrid plasmid vector or a binary vector in trans with a Ti-plasmid possessing a vir region can be used to transform plant cells. Tissue explants such as stems or leaf discs may be inoculated with the bacterium. Alternatively, the bacterium may be co-cultured with regenerating plant protoplasts. Plant protoplasts may also be transformed by direct introduction of DNA fragments which encode the heterologous gene and in which the promoter and appropriate other transcriptional and translational control elements are

present or of a vector incorporating such a fragment. Direct introduction may be achieved using electroporation or polyethylene glycol.

Plant cells from monocotyledonous or dicotyledonous plants can be transformed according to the present invention. Monocotyledonous species include barley, wheat, maize and rice. Dicotyledonous species include tobacco, tomato, sunflower, petunia, cotton, sugarbeet, potato, lettuce, melon, soybean, canola (rapeseed) and poplars. Tissue cultures of transformed plant cells are propagated to regenerate differentiated transformed whole plants. The transformed plant cells may be cultured on a suitable medium, preferably a selectable growth medium. Plants may be regenerated from the resulting callus. Transgenic plants are thereby obtained whose cells harbour the promoter operably linked to the heterologous gene, for example integrated in their genome. The gene is consequently expressible in the cells. Seed from the regenerated plants can be collected for future use.

Expression of the protein encoded by the gene linked to the promoter is determined by the presence or absence of light. Expression occurs when light is present. If desired, therefore, expression of protein can be controlled by artificially increasing or decreasing the length of time for which plants or plant tissue cells are exposed to light, for example in a greenhouse or laboratory.

The following Exampl s illustrate the invention. In

the accompanying drawings:

Figure 1 is a restriction map of FBPase genomic recombinant lambda 6aF1 showing the fragments subcloned for promoter construct preparation and the extent of nucleotide sequencing;

Figure 2 shows the FBPase gene structure;

Figure 3 shows the FBPase upstream sequences with restriction enzyme sites underlined;

Figure 4 shows the construction of plasmid pBIXS, with ▭ denoting FBPase upstream sequences, ▬ denoting FBPase coding sequence, ▭ denoting β-glucuronidase coding sequence and ▭ denoting nopaline synthase terminator sequence;

Figure 5 shows the construction of plasmid pBIES;

Figure 6 shows the construction of plasmid pBIEB;

Figure 7 shows the construction of plasmid pBIHH;

Figure 8 shows the contruction of plasmids pBICH and pBIXH,

Figure 9 shows the FBPase promoter constructs pBIES:ES, pBIXS:XS, pBIHH:HH, pBICH:CH and pBIXH:XH;

Figure 10 shows the sequences at the BamHI and SmaI sites of pBI201.1 and pBI201.2 and the sequences of construct junctions; and

Figure 11 shows the levels of β-glucuronidase activity in transformed plants, the level for untransformed plants being 0.014.

EXAMPLE 1

1. Isolation of a FBPase Gene

The strategy employed to isolate the wheat FBPase promoter involved first constructing a genomic library of wheat DNA and then screening of the library using a cDNA probe for FBPase.

High molecular weight DNA was isolated from dark grown shoots of Triticum aestivum cv Chinese Spring (Lazarus et al, Plant Mol. Biol 5, 8-24, 1985). Conditions for partial digestion with Sau3A were established. DNA fragments of 18-25 Kb were purified by size fractionation on sucrose density gradients. Lambda Charon 35 (Loenen and Blattner, Gene 26, 171-179, 1983) vector DNA was prepared by digestion with BamHI and purification on sucrose density gradients. Vector DNA (1.5 μg) and wheat DNA (3.5 μg) were ligated at high concentration (500 ng/μl) using T4 DNA ligase and subsequently packaged in vitro using commercially available extracts (Stratagene).

About 2.6×10^6 recombinants were plated onto the host E. coli K803 and DNA lifts from the plaques taken onto nitrocellulose filters. The wheat FBPase cDNA (Raines et al, 1988) was random primer labelled using ^{32}PdATP and used to probe the library lifts. Positively hybridizing plaques were purified to homogeneity by several rounds of screening and then DNA from these positive phage was purified for further analysis.

2. Characterization of the FBPase Gene

A restriction map was made of the 14Kb insert DNA of the positive phage 6aF1 (Fig. 1) and the position and orientation of the FBPase gene determined by hybridization studies. DNA fragments covering this region were subcloned into the plasmid vector pUBS1, which is a pUC19 derivative containing the polylinker region of the Bluescript plasmid of Stratagene (Raines et al, 1988). Overlapping sequence was obtained from these clones by exonuclease III digestion and double-stranded dideoxy nucleotide sequence analysis. Data was assembled and analysed using the Staden packages. The extent of the sequence determined is indicated on Fig. 1.

DNA sequence comparisons between the FBPase gene and the cDNA probe used to isolate it revealed no base differences which suggested that the gene copy isolated represents the active progenitor of the cloned mRNA. The FBPase gene structure revealed by these comparisons is shown in Fig. 2.

3. FBPase Upstream Sequences

The regulatory sequences upstream of the FBPase gene, including all of those used in the constructs described below, are given in Fig. 3. This comprises 1726 bp 5′ to the translation initiation codon ATG and 194 bp of protein coding sequence. Restriction enzyme sites used during

subsequent cloning procedures are indicated and underlined. It was proposed that these sequence would contain all of the promoter and other regulatory elements necessary to direct correct expression of protein coding sequences placed downstream under their control.

In order to test this proposal and assess the usefulness of the FBPase promoter in the context of plant transformation a series of constructs were prepared. In each case part of the FBPase upstream region was placed in front of the bacterial reporter gene β-glucuronidase and the nopaline synthase terminator sequences. These expression cassettes were then transferred to a suitable vector for transformation into Nicotiana tabacum. This allowed expression, controlled by the FBPase promoter, to be detected by an enzyme assay for β-glucuronidase activity using a fluorogenic substrate in tissue extracts from transformed plants.

(a) Construction of the plasmid pBIXS containing 497 bp of sequence from 5' of the FBPase translation initiation codon and 44 amino acids of FBPase coding sequence in a translational fusion to β-glucuronidase (Fig. 4)

A 0.6 Kb XbaI-SalI fragment of the FBPase gene (these sites are shown in Fig. 3) was subcloned into the plasmid vector pUBS1 to form the plasmid p0.6XS. p0.6XS was linearised using SalI and the site filled in using the

Klenow fragment of E. coli DNA polymerase I. The insert was then removed by digestion with XbaI and the resulting fragment subcloned into SmaI-XbaI digested pBI201.1 (Jefferson, Plant. Mol. Biol. Reporter 5, 387-405, 1987). Plasmid pBI201.1 is a promoterless β-glucuronidase cassette vector. Ligation resulted in the formation of pBIXS. The junction of FBPase sequences was checked by nucleotide sequencing (Fig. 10).

(b) Construction of the plasmid pBIES containing 1726 bp of sequence from 5' of the FBPase translational initiation codon and 44 amino acids of coding sequence in a translational fusion to β-glucuronidase (Fig. 5)

A 1.8 Kb EcoRI-SalI fragment of the FBPase gene (Fig. 3) was subcloned into the plasmid vector pUBS1 to form the plasmid p1.8ES.p1.8ES was linearised using SalI and the site filled in using the Klenow enzyme before digestion with EcoRI. This FBPase gene fragment was then reinserted into SmaI-EcoRI cut pUBS1 vector. The resulting plasmid p1.8Eb was digested with BamHI and SalI and the fragment ligated into BamHI-SalI cut pBI201.2 (Jefferson, 1987) to create the construct pBIES. The sequence of the fusion junction is shown in Fig. 10.

(c) Construction of the plasmid pBIEB, a transcriptional fusion to the β-glucuronidase coding sequence of 1726 bp of FBPase promoter sequence (Fig. 6)

This construct was designed to provide additional restriction enzyme sites to allow future modifications to be made to the promoter sequences. The plasmid p1.8ES was digested with BamHI and SalI and the FBPase fragment released purified by agarose gel electrophoresis. This fragment was then digested with HhaI and the overhang removed by digestion with T4 DNA polymerase. Following EcoRI digestion the fragment was ligated into SmaI-EcoRI cut pUBS1. The resulting plasmid p1.8EH was digested with KpnI and the site blunted using T4 DNA polymerase, followed by digestion with BamHI. The vector pBI201.1 was digested with SalI and the site filled in using the Klenow enzyme before cutting with BamHI. Vector and FBPase promoter fragment were then ligated together to create the plasmid pBIEB. The sequence of the fusion junction is shown in Fig. 10.

(d) Construction of the plasmid pBIHH containing 1726 bp of FBPase promoter sequence upstream of β-glucuronidase (Fig. 7)

p1.8Eb was digested with SalI and BamHI and the FBPase promoter fragment purified by agarose gel electrophoresis. The fragment was then digested with HhaI and the overhang blunted using T4 DNA polymerase before HindIII digestion. The FBPase promoter was then ligated

into HindIII-SmaI cut pBI201.1 to give the construct pBIHH. The sequence of the 3' promoter junction is shown in Fig. 10 and is identical to that in pBICH and pBIXH constructs described below.

(e) Construction of the plasmids pBICH and pBIXH containing 881 bp and 444 bp respectively of FBPase promoter sequence upstream of β-glucuronidase (Fig. 8)

These constructs are based on pBIHH (described above) but contain less promoter sequence and have additional polylinker sites 5' to the FBPase sequence. pBIHH and pBIEB were both digested with ClaI and EcoRI and the vector portion of pBIEB ligated to the FBPase-β-glucuronidase fragment derived from pBIHH. This produced the construct pBICH. Digestion of pBICH with XbaI and ClaI followed by filling in with the Klenow enzyme and religation created the plasmid pBIXH. E. coli MC 1022 harbouring p1.8ES was deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, GB on 25 August 1989 under accession number NCIMB 40183.

4. Transfer of FBPase promoter fusions into binary vectors and introduction into Nicotiana

The FBPase promoter constructs that were obtained are shown schematically in Figure 9. The FBPase promoter β-glucuronidase expression cassettes were subcloned from their pUC19 derived vectors as EcoRI-HindIII (pBIXS, pBICH,

pBIXH) or EcoRI (pBIES, pBIEB, pBIHH) fragments into the binary vector pBIN 19 (Bevan, Nucl. Acids Res. 12, 8711-8721, 1984). These constructs were then mobilised from Escherichia coli MC1022 into Agrobacterium tumefaciens LBA4404 as described (Bevan, 1984). Leaf discs of Nicotiana tabacum var. Samsun were transformed as described (Horsch et al, Science 223, 496-498, 1984) and selected on shooting medium containing 100 μg/ml kanamycin.

5. β-glucuronidase assay of transformed plants

The activity of the FBPase promoter in individual light-grown transformants was determined by measuring β-glucuronidase activity in leaf extracts. Tissue extracts were prepared and analysed for fluorescence of the reaction product 4-methyl umbelliferone as described (Jefferson, 1987). Reactions were usually incubated at 37°C for 4 hours with aliquots sampled at 60 min intervals. The protein concentration in each extract was measured to allow direct comparisons to be made between them (using a Bio-Rad kit).

The two translational fusions (pBIXS and pBIES) and one transcriptional fusion (pBIEB) yielded no plants with measurable β-glucoronidase activity above that of untransformed controls. Between 5 and 10 plants containing each construct were regenerated and assayed.

Three of the transcriptional fusions, namely pBIHH, pBICH and pBIXH did give significant levels of β-glucuronidase activity in transformed plants. The results

of these assays expressed in nmol 4-methyl umbelliferone produced/min/mg protein are shown in Fig. 11. Furthermore, even given the variation commonly observed between individual transformed plants, there is a significant drop in activity when the length of upstream sequence is reduced to the 444 bp present in the construct pBIXH. Plasmids pBIHH and pBICH are therefore vectors according to the invention. The level of activity in untransformed plants was 0.014.

EXAMPLE 2: Light regulation of the FBPase promoter in transformed tobacco plants

Seeds from the transformed tobacco plant "HH18" were plated on tissue culture medium for germination. HH18 was one of the four pBIHH tobacco transformants from Example 1 and, in particular, was plant 1 from Figure 11. These F1 seeds were a heterogeneous population representing all possible genetic combinations, as more than one copy of the construct may have inserted into the genome. Due to the mixed nature of the F1 generation plants 6-8 seedlings given each treatment were assayed for β-glucuronidase activity.

The average β-glucuronidase activity of light-grown 8 day seedlings was 2.075 pmol 4-methyl umbelliferone formed/min/5μl extract and for etiolated (dark-grown) plants was 0.126. In the same experiment the value for untransformed tobacco was 0.025.

CLAIMS

1. A light-activatable promoter having the sequence:

ATCGATAGGTTCGACAGAAGCTGCCACGTACCTATGAAACTCAATTGATTATTATATCCA
TATTATTGAAGTTTATTTTTGTAGAATGTTATTCAATTCCAGAAGTTGTAAGTTCCGTAA
GACCTGTAATATTGGCCCAATCGAAGACCAACGTTTCTTGAGCTAAATTTAGCTTTTTTT
TTAGAAAGTAATTTAGCTTGCACCCTGGTAAGTGCCACAGAGTGGCACCAATACATGGAA
CTCAAACATTTTTTGTCCCAGGTTTAGTGACGCGATGATGACAGCTTTAGTTGTCAAGCA
TGACAACCTTTTTGAATGGTAAGTTTTACTCTCTTTTTTAGATGACAACTTAGTTTTTAA
AAAGTCAGGCCAAAGTGCTTCGTGACACACGTGTGACACTTATCATTCAGTTTGTCTAAT
TCACATCTAGATATTTTTTAAGGATGTCACATCTAACCTCCCACAAGTATATAATGCATC
AATAAGAAACAAAAAAAACTAGGACAAAAAAATAGACCACAAACAGAGTGAAAATCAGTT
TAGATATGACATAACTATGTCACATTTAGATGTGTCCTAGACAGACCCCTTATCATTTGG
GTTTACCTTTAGTAGCGGGCGTATGCCGCGGCATCAATAAATCATCATCATGTAATGTAT
ATAAGGCGTTTAGAGACTTGACGAAGGTTGTATCTACGATCCACGAAATATCTAATCTCC
AAAGGTAGGTGAATCAGACTGAAGCGAGCGACCACATCCTCACAATTCTGCCCCAATCCA
CACATTCGCCTCCAGCCCTCTCTCAAGCCACACAAACCGAGCCCGGAACCAATGGAACAA
AACAAGAAGCCGGCACCACCACCACGGTG

optionally modified by one or more base substitutions, insertions and/or deletions and/or by an extension at either or each end provided that the thus-modified sequence is capable of acting as a light-activatable promoter.

2. A promoter according to claim 1, having the sequence:

GAATTCTAAGGATGGGCATGGGAGGGGGTGGGGTTTCAACCTGAGGATTTTTTCCAACCT

AAATACAGACTATGCAAACTACCTATTGTGTTTTATTCAAAAATACATCCCTTGCCCAAA

ACGAGGTAGGGCAACACCTATAGATGGCTATGGAAGAAGTTATGGAGTATCATAATATAT

GTTTCATCATATCCTTCCATATGTTATCATGATAACTTTAAAGTCCTGAAATTTGCGATA

TGCACTTGAAGCTCCGCTGTCATTCCCATGGCTTAAAAGTCACTGATTGGAGAGCATGTT

GTGTTTTTGCCCGTAGATCCATTGGCTTTAAGGTTTGATAGGTCATCGCCCTATGCTTTA

ACCTATCATGACAATAGTGGCAACAAATGCGGAAATCCAACTCGGTGCCCAGGCTCATCT

GCTCTCGGTCAGAAAAAAAATCAAAACAAATACTAGAAAAATACAAACCCCGATTTGTCT

TTTTTGCCGAGAGCTACTCAGATGTCCAAATGAGTTGAACTTTAGAACGGACCTACGTAT

CGAATTATCTACCACACATTTTTTTTTAATTTTTCTAGTATTTGTTATGATTTTTTTGTC

AGTGCACCTAAGCCCGGGAGCAGAAACGCCGCGTCCCAACAAATGCTAATGTTACGTTTG

ATTTGGTAAATGGTTCTCTTTGTGTTGTGTATCTTTTGGTTGTATTAGAAATCTTTGTGG

TCCATATGTGCATAATTTGTTCAATAAATCAATAATGTCAGATCGTCCTAAAATAAACTT

GAGAAGAAATTCATCGATAGGTTCGACAGAAGCTGCCACGTACCTATGAAACTCAATTGA

TTATTATATCCATATTATTGAAGTTTATTTTTGTAGAATGTTATTCAATTCCAGAAGTTG

TAAGTTCCGTAAGACCTGTAATATTGGCCCAATCGAAGACCCAAGTTTCTTGAGCTAAAT

TTAGCTTTTTTTTTAGAAAGTAATTTAGCTTGCACCCTGGTAAGTGCCACACGAGTGGCA

CCAATACATGGCAACTCCAAACATTTTTTGTCCCAGGTTTAGTGACGCGATGATGACAGC

TTTAGTTGTCAAGCATGACAACCTTTTTGAATGGTAAGTTTTACTCTCTTTTTTGAGTTT

TTTAGATGACAACTTAGTTTTTAAAAAGTCAGGCCAAAGTGCTTCGTGACACACGTGTGA

CACTTATCATTCAGTTTGTCTAATTCACATCTAGATATTTTTTAAGGATGTCACATCTAA

CCTCCCACAAGTATATAATGCATCAATAAGAAACAAAAAAAACTAGGACAAAAAAATAGA

CCACAAACAGAGTGAAAATCAGTTTAGATATGACATAACTATGTCACATTTAGATGTGTC

CTAGACAGACCCCTTATCATTTGGGTTTACCTTTAGTAGCGGGCGTATGCCGCGGCATCA

ATAAATCATCATGTAATGTATATAAGGCGTTTTAGAGACTTGACGAAGGTTGTATCTACG

ATCCACGAAATATCTAATCTCCAAAGGTAGGTGAATCAGACTTAAGCGAGCGACCACATC

CTCACAATTCTGCCCCCAATCCACACATTCGCCTCCAGCCCTCTCTCAAGCCACACAAAC

CGCAGCCCGGAACCAATGGAACAAAACAAGAAGCCGGCACCACCACCACGGTGC.

optionally modified as defined in claim 1.

3. A DNA fragment comprising a promoter as claimed in claim 1 or 2 operably linked to a heterologous gene encoding a protein.

4. A vector which comprises a heterologous gene, encoding a protein, under the control of a promoter as claimed in claim 1 or 2, such that the gene is capable of being expressed in a plant cell transformed with the vector.

5. A vector according to claim 4, wherein the promoter is fused directly to the 5'-end of the said gene.

6. A vector according to claim 4 or 5, which further contains a region which enables the gene and the promoter to be transferred to and stably integrated in a plant cell genome.

7. A vector according to any one of claims 4 to 6, which is a plasmid.

8. A plant cell which has been transformed with a vector as claimed in any one of claims 4 to 7.

9. A plant cell which harbours a promoter as claimed in claim 1 or 2 operably linked to a heterologous gene encoding a protein.

10. A transgenic plant which has been regenerated from plant cells as claimed in claim 8 or 9.

11. A transgenic plant which harbours in its cells a promoter as claimed in claim 1 or 2 operably linked to a heterologous gene encoding a protein.

12. Seed obtained from a transgenic plant as

claimed in claim 10 or 11.

13. A method of producing a desired protein in a plant cell, which method comprises:

(i) transforming a plant cell with a vector as claimed in any one of claims 4 to 7, the protein encoded by the gene under the control of the said promoter being the desired protein; and

(ii) culturing the transformed plant cell under conditions of light which allow expression of the protein.

14. A method of producing a transgenic plant capable of producing a desired protein, which method comprises:

(i) transforming a plant cell with a vector as claimed in any one of claims 4 to 7, the protein encoded by the gene under the control of the said promoter being the desired protein; and

(ii) regenerating plants from the transformed cells.

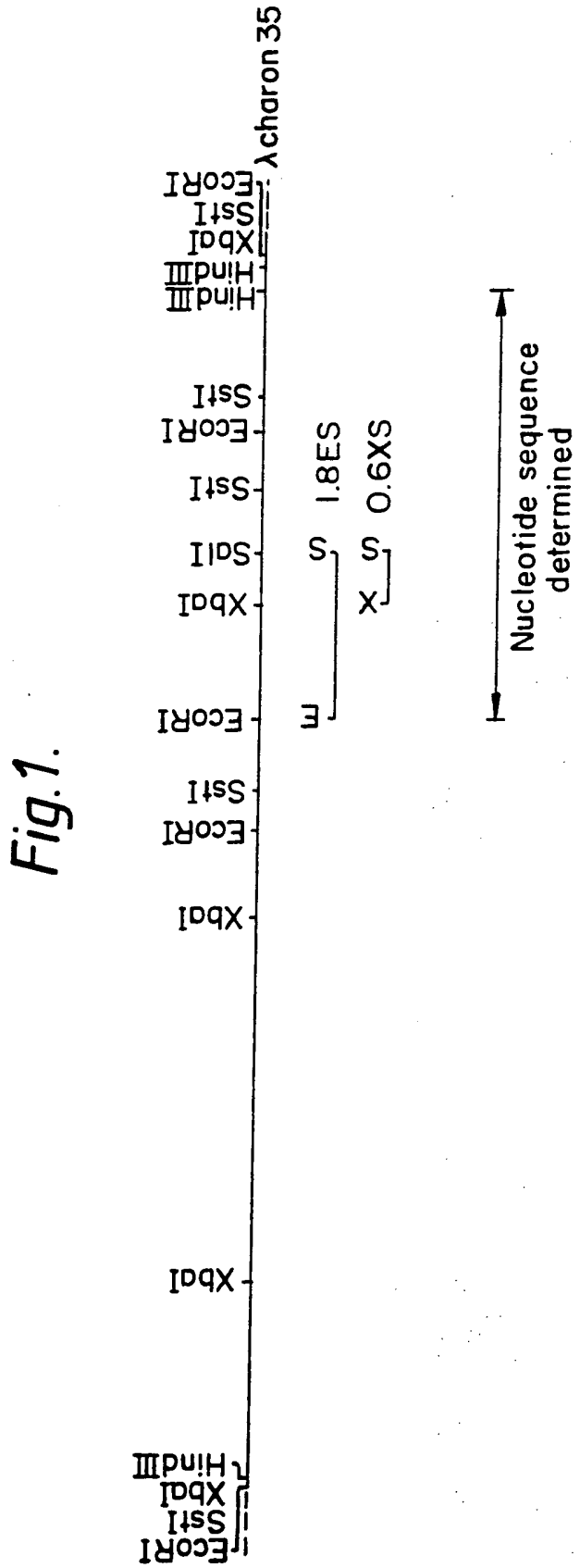

λcharon 35
EcoRI
SstI
XbaI
HindIII
HindIII
SstI
EcoRI
SstI
SalI
XbaI
EcoRI
SstI
EcoRI
XbaI
XbaI
HindIII
XbaI
SstI
EcoRI
1.8ES
0.6XS
E
S
X
S
Nucleotide sequence determined

Fig. 1.

Fig. 2.

fructose-1, 6-bisphosphatase gene

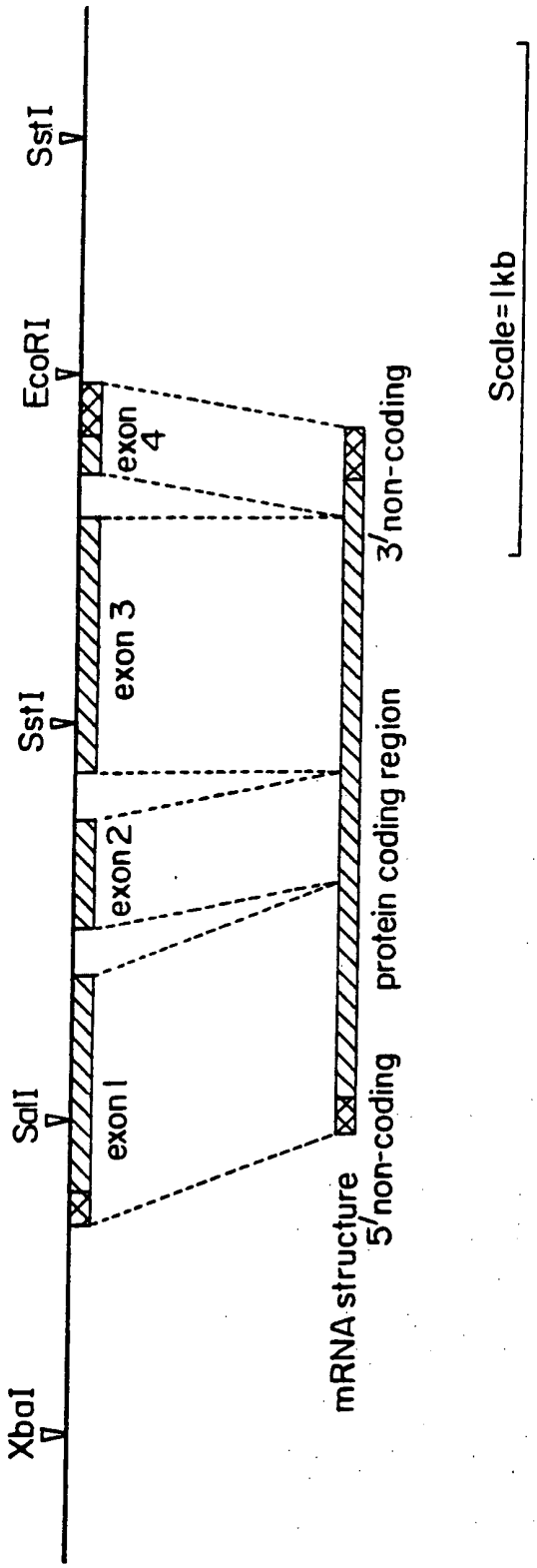


Fig. 3.

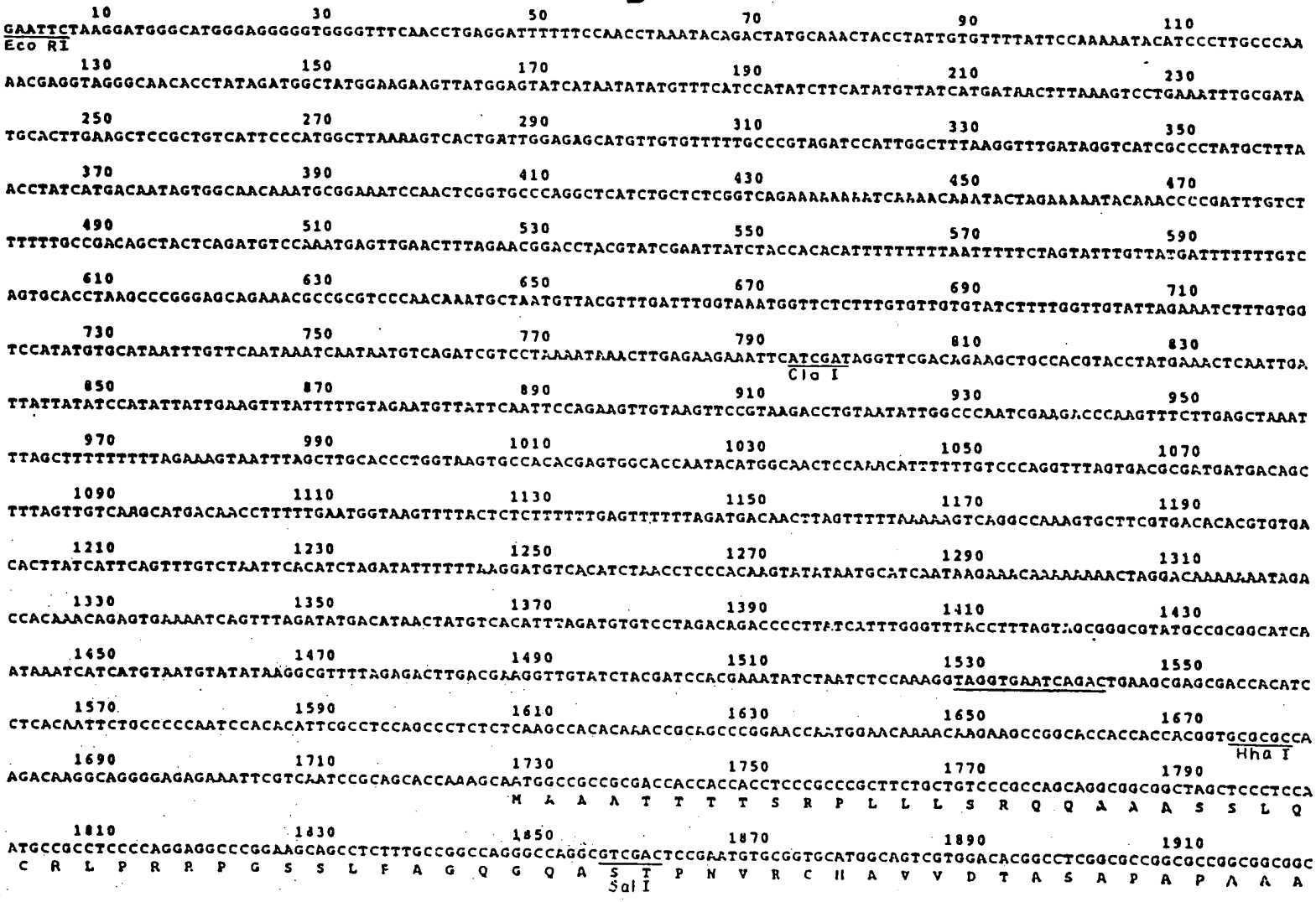


Fig.4.

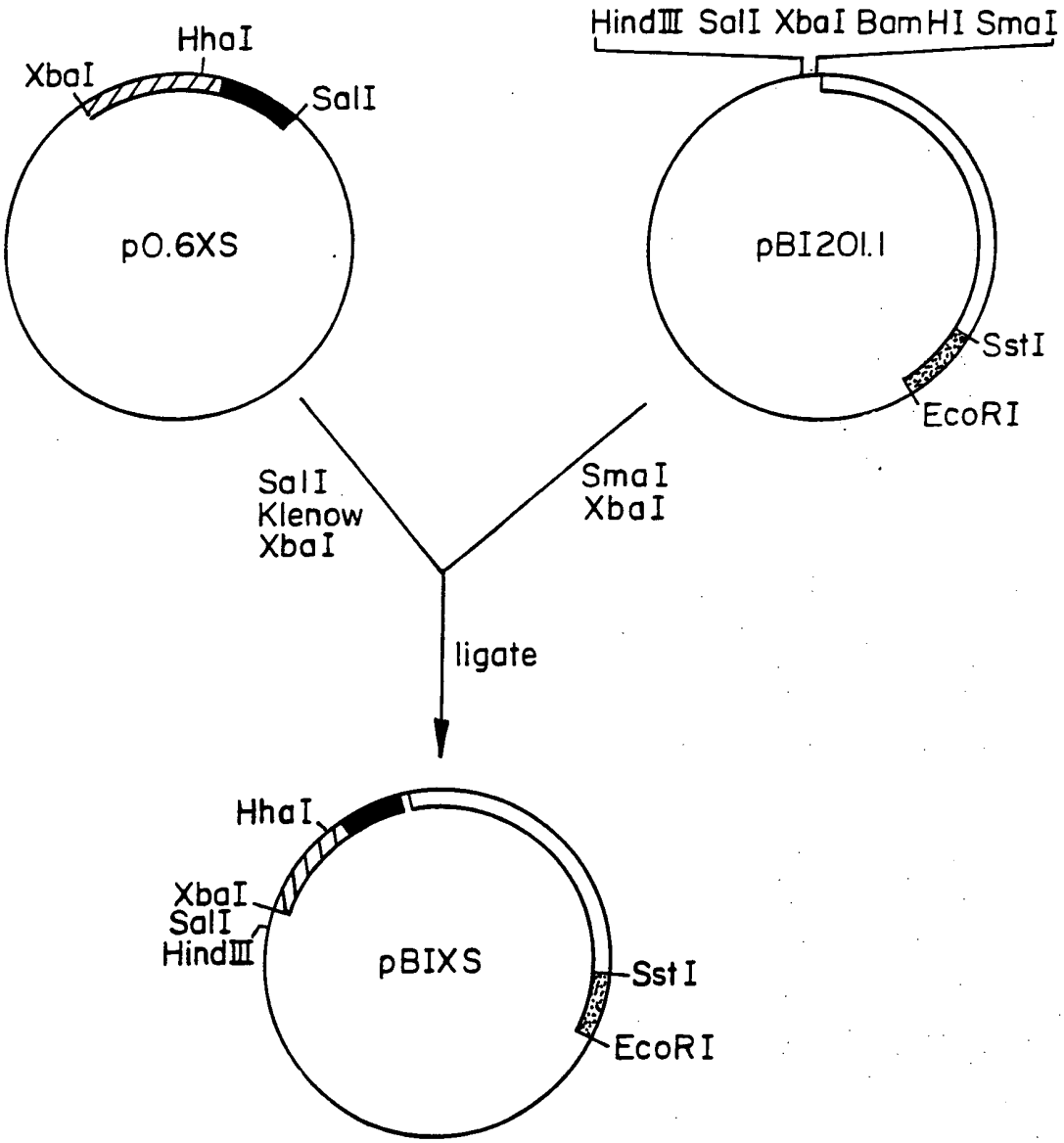
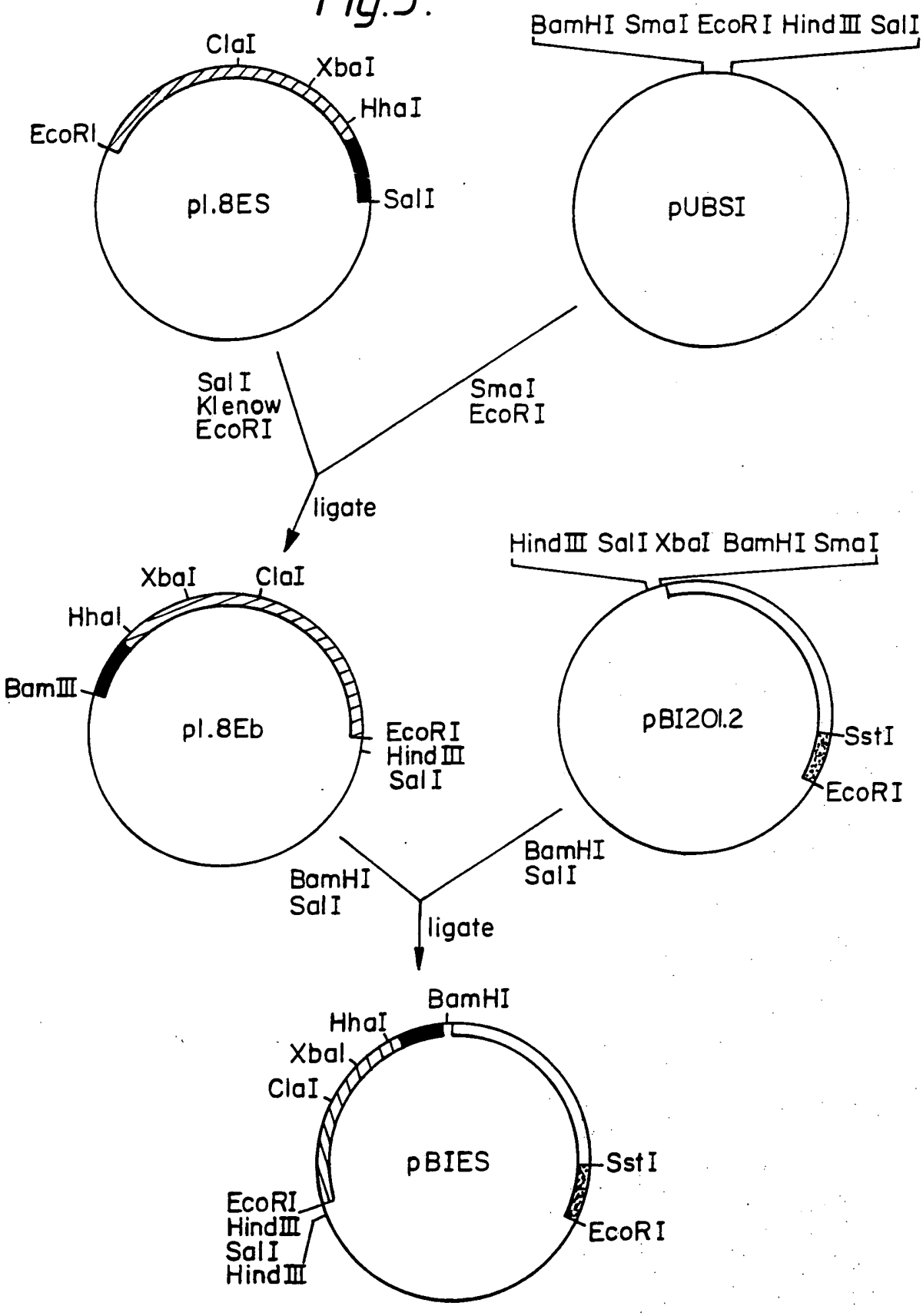
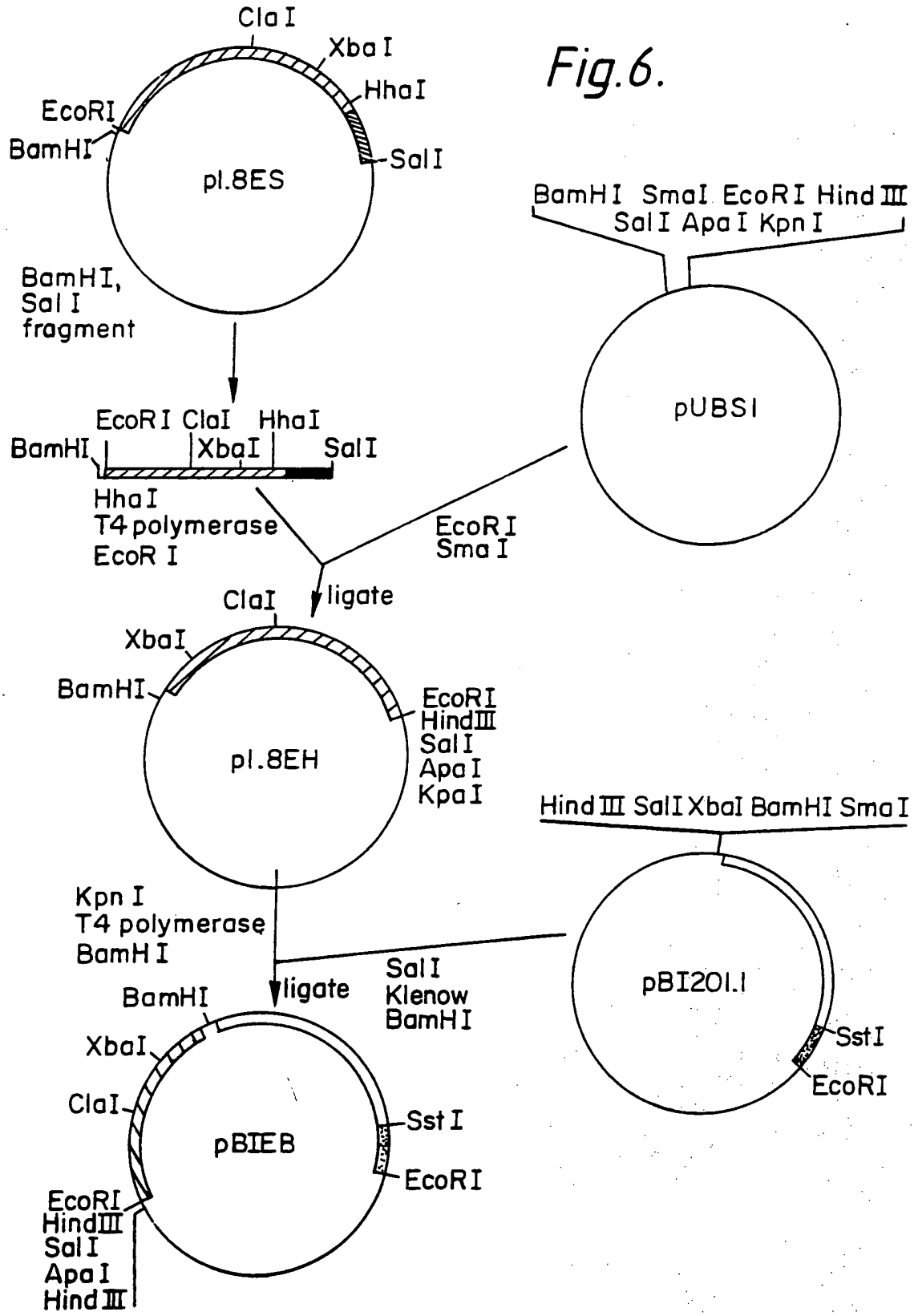

HhaI
XbaI
SalI
pO.6XS
HindIII SalI XbaI BamHI SmaI
pBI201.1
SstI
EcoRI
SalI
Klenow
XbaI
SmaI
XbaI
ligate
HhaI
XbaI
SalI
HindIII
pBIXS
SstI
EcoRI

Fig.5.



SUBSTITUTE SHEET

Fig.6.



SUBSTITUTE SHEET

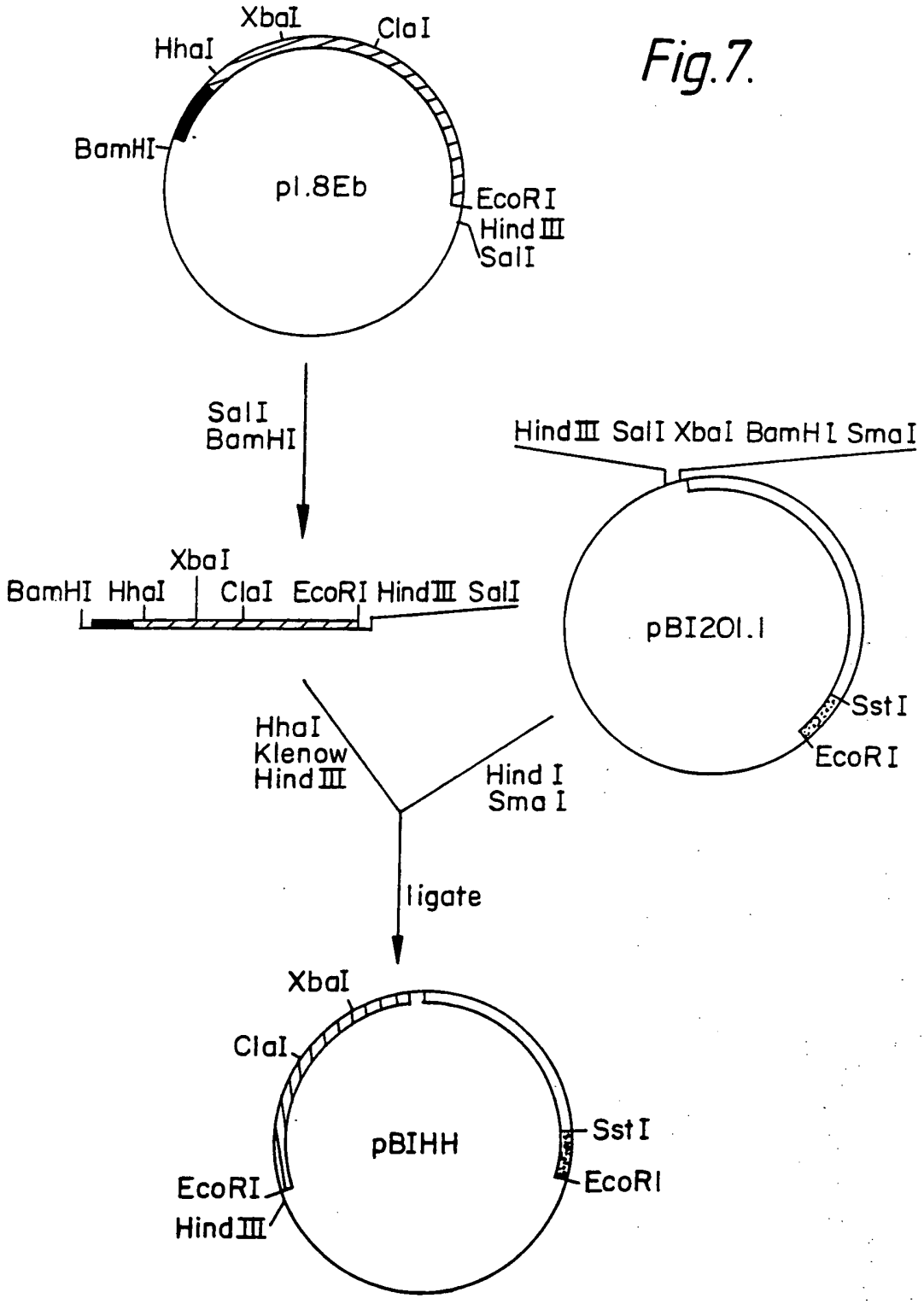

XbaI
ClaI
HhaI
BamHI
pI.8Eb
EcoRI
Hind III
SalI
SalI
BamHI
Hind III SalI XbaI BamH I SmaI
XbaI
BamHI HhaI
ClaI EcoRI Hind III SalI
pBI201.1
SstI
EcoR I
HhaI
Klenow
Hind III
Hind I
Sma I
ligate
XbaI
ClaI
pBIHH
SstI
EcoRI
EcoRI
Hind III

Fig.7.

Fig.8.

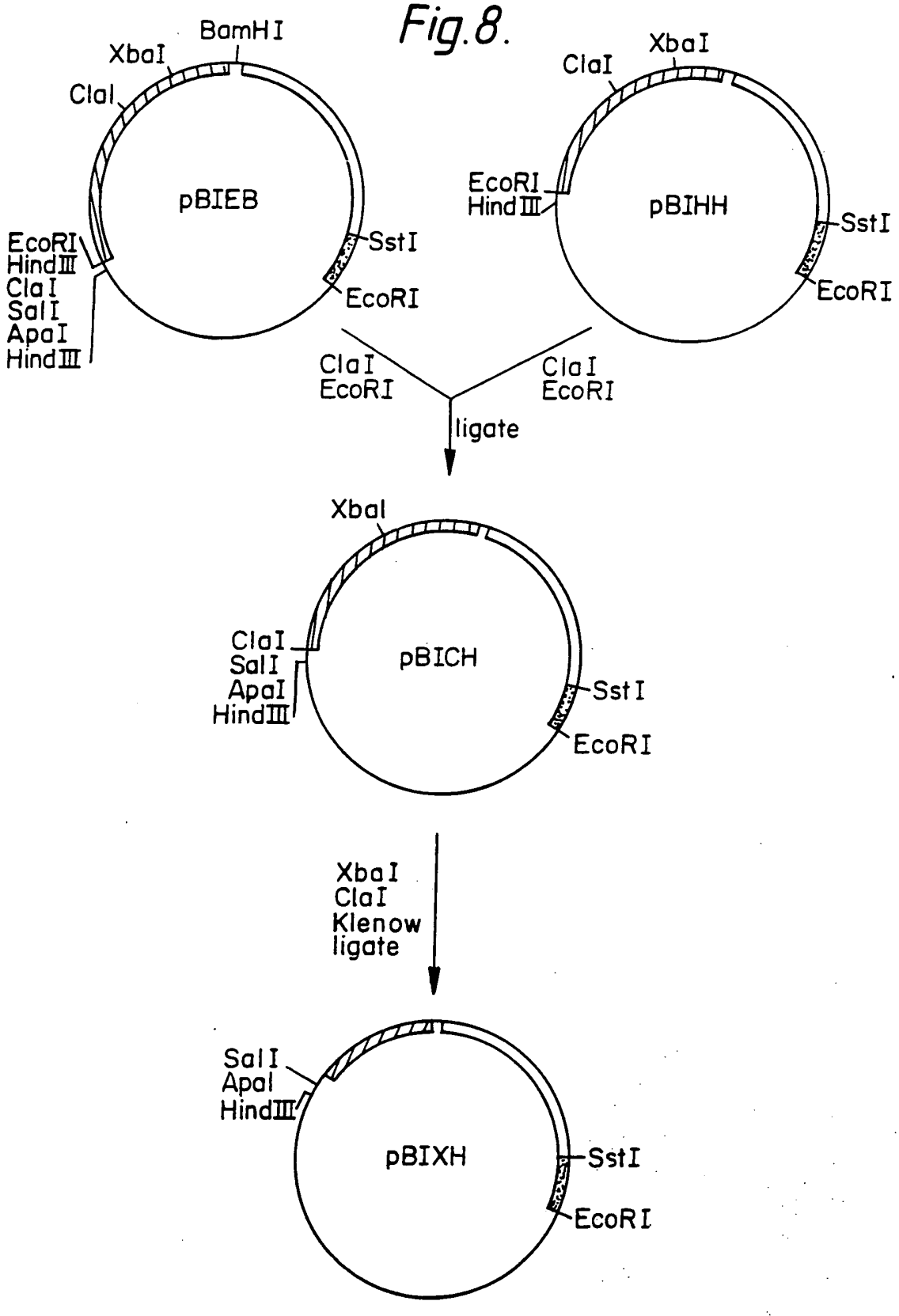

BamHI
XbaI
ClaI
pBIEB
EcoRI
HindIII
ClaI
SalI
ApaI
HindIII
SstI
EcoRI
XbaI
ClaI
pBIHH
EcoRI
HindIII
SstI
EcoRI
ClaI
EcoRI
ClaI
EcoRI
ligate
XbaI
pBICH
ClaI
SalI
ApaI
HindIII
SstI
EcoRI
XbaI
ClaI
Klenow
ligate
SalI
ApaI
HindIII
pBIXH
SstI
EcoRI

Fig. 9.

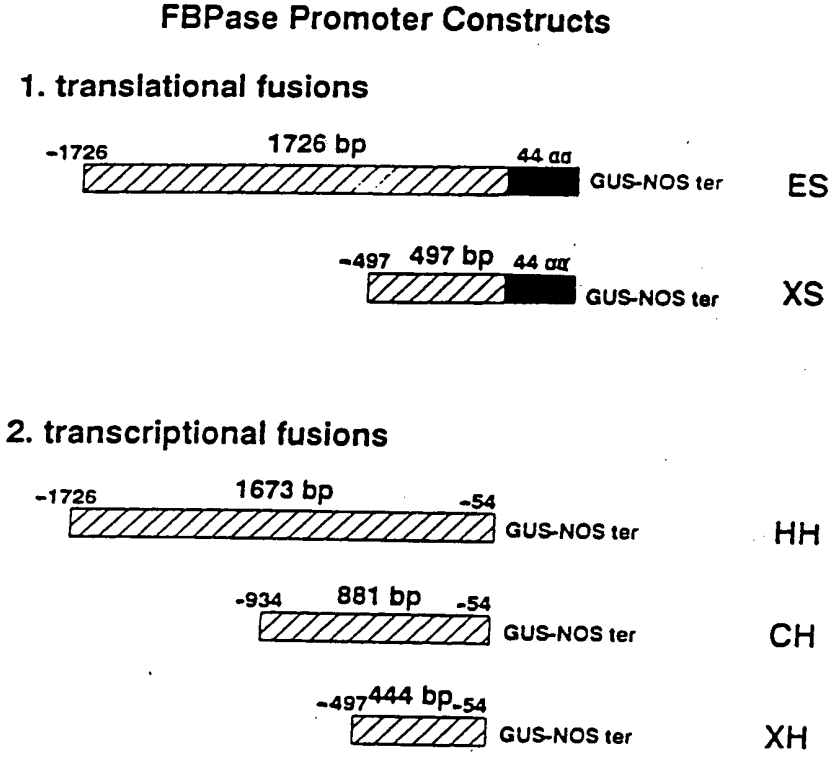
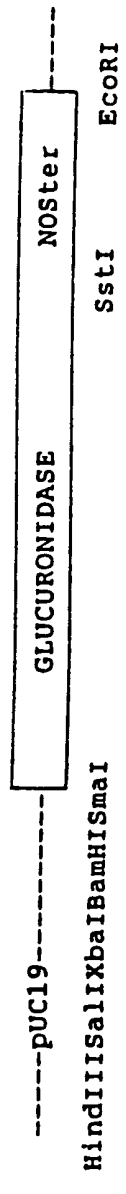

FBPase Promoter Constructs
1. translational fusions
-1726
1726 bp
44 aa
GUS-NOS ter
ES
-497
497 bp
44 aa
GUS-NOS ter
XS
2. transcriptional fusions
-1726
1673 bp
-54
GUS-NOS ter
HH
-934
881 bp
-54
GUS-NOS ter
CH
-497
444 bp
-54
GUS-NOS ter
XH

Fig.10.



pBI201.1 GGATCCCCGGGTGGTCAGTCCCTTATG
BamHI SmaI

pBI201.2 GGATCCCCGGGTAGGTCAGTCCCTTATG
BamHI SmaI

Sequences of construct junctions (FBPase sequence in bold)

pBIES GTCGAGGGGGATCCCCGGGTAGGTCAGTCCCTTATG
BamHI

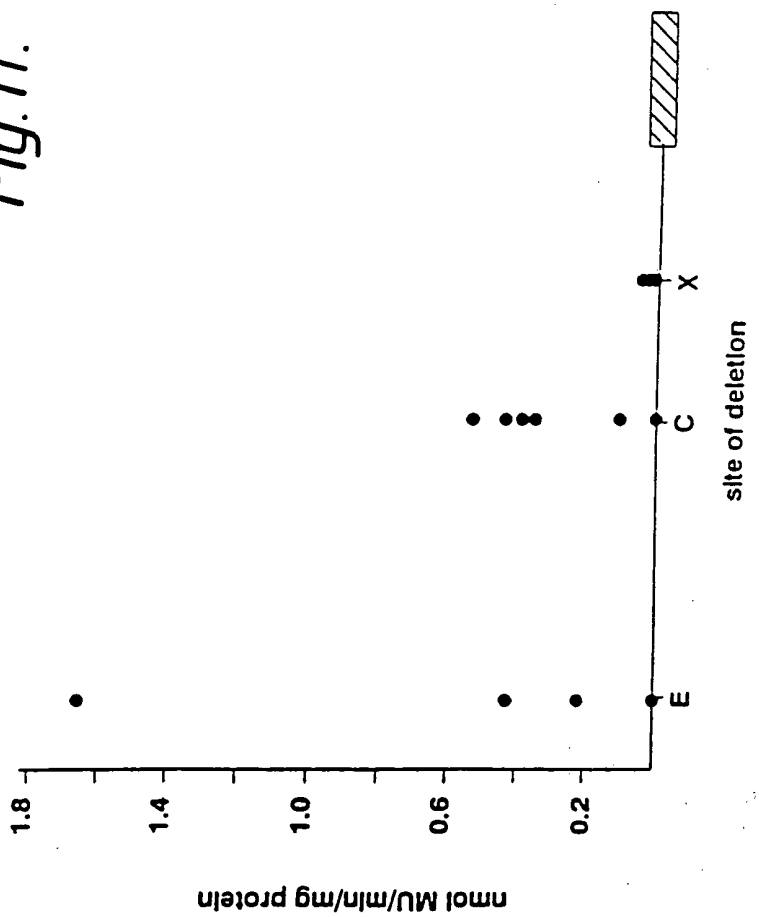
pBIXS GTCGAGGGTGGTCAGTCCCTTATG

pBIEB CCACGGTGGGGGGATCCCCGGGTGGTCATCCCTTATG
BamHI

pBIHH
pBICH
pBIXH CCACGGTGGGGTGGTCAGTCCCTTATG

Fig.11.

	(EcoRI)	(ClaI)	(XbaI)
1	1.648	0.538	0.042
2	0.442	0.538	0.042
3	0.231	0.385	0.023
4	0.015	0.359	-
5	-	0.125	-
6	-	0.011	-
7	-	0.011	-



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01493

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/82, C 12 N 15/55, C 12 N 5/10, A 01 H 5/00, A 01 H 5/10, C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, A 01 H, C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Nucleic Acids Research, vol. 16, no. 16, 1988, IRL Press Limited, (Oxford, GB), C.A. Raines et al.: "Chloroplast fructose-1,6-bisphosphatase: the product of a mosaic gene", pages 7931-7942, see the whole article (cited in the application) --	1-14
A	Mol. Gen. Genetics, vol. 218, 1989, Springer-Verlag, S. Chao et al.: "Chromosomal location and copy number in wheat and some of its close relatives of genes for enzymes involved in photosynthesis", pages 423-430, see the whole article ----------	1-14

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6th December 1990	23 JAN 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER